Abstract. The thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NCC), which is expressed on the apical membrane of epithelial cells lining the distal convoluted tubule, is responsible for the reabsorption of 5% to 10% of filtered Na\textsuperscript{+} and Cl\textsuperscript{−}. To date, functional studies on the structural and regulatory requirements for localized trafficking and ion-transporting activity of NCC have been hampered by lack of a suitable cell system expressing this cotransporter. Reported here is the functional expression of human NCC (hNCC) in a polarized mammalian cell of renal origin—that is, the high-resistance Madin-Darby canine kidney (MDCK) cell. Western blot testing revealed that the cells predominantly expressed the complex glycosylated (approximately 140 kD) form of hNCC. hNCC was present primarily in the apical part of the cell. The functionality of hNCC was demonstrated by the gain of thiazide-sensitive Na\textsuperscript{+} uptake and transepithelial transport activity. Na\textsuperscript{+} uptake was significantly increased after short-term (15 min) treatment with forskolin, whereas cyclic guanosine monophosphate, wortmannin, phorbol 12-myristate 13-acetate, and staurosporine were without effect. This indicates that hNCC activity is regulated through cyclic adenosine monophosphate, rather than via cyclic guanosine monophosphate, phosphoinositide 3-kinases or protein kinase C. Aldosterone did not alter Na\textsuperscript{+} uptake in the short term (15 min) but significantly increased the transport activity in the long term (16 h). The latter effect of aldosterone was due to an effect on the cytomegalovirus promoter/enhancer driving the expression of hNCC. hNCC-MDCK cells are a good model for the study of the regulation of apical trafficking and ion-transporting activity of hNCC.

The mammalian thiazide-sensitive Na\textsuperscript{+}-Cl\textsuperscript{−} cotransporter (NCC) is expressed in the distal convoluted tubule (DCT), where it is responsible for the reabsorption of 5% to 10% of filtered Na\textsuperscript{+} and Cl\textsuperscript{−} (1). NCC is the site of action of the thiazide diuretics, which are of particular therapeutic relevance for the treatment of hypertension (2,3). These antihypertensive drugs act by inhibiting NCC transport activity, thus promoting the excretion of Na\textsuperscript{+} and Cl\textsuperscript{−}. Loss-of-function mutations in NCC have been shown to cause Gitelman syndrome (OMIM 263800), a disease characterized by salt wasting, hypokalemic metabolic alkalosis, hypomagnesemia, and hypocalciuria (4). Mutations include missense, frameshift, nonsense, and splice-site mutations. Gitelman syndrome shows considerable phenotypic variability. Thus far, no correlation between specific mutations and particular phenotypes has been reported.

NCC belongs to the family of electroneutral cation-chloride cotransporters (CCC) (5,6), which also comprises two Na\textsuperscript{+}-K\textsuperscript{+}-2Cl\textsuperscript{−} cotransporters (NKCC1 and NKCC2) (7,8) and at least four K\textsuperscript{+}-Cl\textsuperscript{−} cotransporters (KCC1 to 4) (9). Genes encoding the various CCC show substantial sequence homology and hydrophy analysis revealed a common predicted topology of 12 membrane-spanning domains, a large extracellular loop with potential N-linked glycosylation sites, and amino and carboxy termini located within the cytoplasm (10). The CCC perform a wide variety of physiologic functions, including cellular volume regulation, vectorial movement of Cl\textsuperscript{−}, and cations across epithelia and control of the extra- and intracellular ionic environments of neurons. This versatility in function reveals itself differences in patterns of tissue expression and cellular localization (11). NCC is present on the apical membranes of DCT (12), whereas NKCC2 is apically expressed in the thick ascending limb of the loop of Henle (13).

Whole-animal studies have implicated the mineralocorticoid hormone aldosterone and the sex hormone estradiol in the regulation of NCC expression. Evidence for a regulatory role of aldosterone comes from several observations. Thus, dietary NaCl restriction, which is known to potently stimulate aldosterone secretion, markedly increased thiazide-sensitive Na\textsuperscript{+} reabsorption by the DCT (14). Furthermore, treatment with aldosterone significantly increased the number of thiazide-binding sites in renal cortical membrane (15,16). Finally, NCC
protein expression was strongly upregulated in rats treated with aldosterone for 10 d (17). A role of sex hormones in the regulation of NCC expression was suggested by the observation that the thiazide receptor density was twofold higher in female rats than in male rats (18). More recently, it was demonstrated that ovariectomy decreased NCC expression and that estradiol replacement fully restored this effect (19).

In addition to the whole-animal studies discussed above, regulation of NCC function and expression was also studied in *Xenopus laevis* oocytes. By use of this expression system, it was found that neither cyclic adenosine monophosphate (cAMP) nor cyclic guanosine monophosphate (cGMP) affected Na+ uptake mediated by flounder NCC (11). On the other hand, the phorbol ester phorbol 12-myristate 13-acetate (PMA), which is a potent activator of protein kinase C (PKC), markedly decreased Na+ uptake by oocytes expressing either flounder (20) or rat (21) NCC. It was not investigated, however, whether PKC decreased the activity or the plasma membrane expression of NCC. Although these studies provide important new insights into the regulation of NCC expression and function, a major drawback of the oocyte expression system is that it lacks the physiologic background and polarized structure of the native epithelial kidney cell. This urged us to stably express human NCC (hNCC) in polarized Madin-Darby canine kidney (MDCK) cells.

Thus far, NKCC1 is the only one of the CCC that has been studied in a polarized cell. To this end, human NKCC1 was stably expressed in the low K+ resistant mutant MDCK cell line LK-C1 (22). Regulation of human NKCC1 proved to be similar to that in the native secretory cell, as it was activated by cell shrinkage, calyculin A, and low Cl− (23). The data presented here show that we successfully generated a MDCK cell line stably expressing hNCC at the apical membrane. The cells were used to assess the involvement of several signaling pathways in the regulation of membrane localization and transport activity of this Na+−Cl− cotransporter.

**Materials and Methods**

**Contracts**

To obtain DNA encoding hNCC, the oocyte-expression vector pT7-T1-hNCC was digested with *Bam*HI and *Spe*I. The generation of the pT7-T1-hNCC vector has been described previously (24). Subsequently, the *Bam*HI/*Spe*I fragment was ligated into the *Bgl*II/XbaI sites of the mammalian vector pCB6. Transcription of hNCC is driven by the cytomegalovirus promoter.

**Culturing, Transfection, and Selection of Clones**

High-resistance MDCK (MDCK-HRS) and MDCK-AQP2-EK were grown in DMEM supplemented with 5% (vol/vol) FCS at 37°C in 5% CO2. The MDCK-AQP2-EK cell line was provided by PM Deen, Cell Physiology, University Medical Centre Nijmegen. MDCK-HRS cells were transfected with 25 to 30 μg of circular pCB6-hNCC by means of calcium-phosphate precipitation technique as described in detail elsewhere (25). Twenty-four hours after transfection, the cells were trypsinized, divided over 6 to 8 petri dishes of 57 cm2, and cultured in DMEM containing 800 μg/ml G418 (Life Technologies Europe, Breda, The Netherlands). Ten to 14 d after transfection, individual colonies were selected by means of cloning rings and expanded. After eight passages after selection of a clone, G418 was omitted from the medium.

**Immunoblotting**

The cells were grown to confluence in a humidified atmosphere of 95% air, 5% CO2, at 37°C, and lysed in 200 μl Laemmli buffer containing 50 mM Tris/HCl (pH 6.8), 2% (w/v) SDS, 12% (vol/vol) glycerol, 0.01% (w/v) bromophenol blue, and 25 mM dithiothreitol (DTT) for 30 min. Proteins were separated on a 6% (w/v) SDS-polyacrylamide gel and transferred electrophoretically to PVDF membranes (Millipore, Bedford, MA). Blots were incubated with either rabbit anti-hNCC antibody A857 (26) or affinity-purified rabbit AQP2 (27) diluted 1:10,000 or 1:3000 in PBS buffer supplemented with 5% (w/v) nonfat dried milk, respectively. Subsequently, blots were incubated with sheep horseradish peroxidase conjugated to anti-rabbit IgG (Sigma, St. Louis, MO) diluted 1:5000. Finally, proteins were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

**Immunocytochemistry**

Cells were seeded at a density of 3 × 105 cells/cm2 on 0.33 cm2 filters and grown to confluence for 2 d. Next, the cells were rinsed with ice-cold PBS and fixed in PBS containing 3% (w/v) paraformaldehyde at room temperature. After three washes with PBS, the cells were incubated in PBS containing 50 mM NH4Cl for 15 min. Cells were washed with PBS containing 1 mM MgCl2 and 0.1 mM CaCl2 and incubated in PBS with 0.1% (w/v) BSA (blocking buffer) for 20 min. Subsequently, cells were washed in PBS, and filters were cut from their plastic support. Filters were incubated overnight with 25 to 30 μl anti-hNCC antibody A857 (26) diluted 1:6000 in PBS containing 0.05% (w/v) saponin. After three washes with PBS, the filters were incubated with 30 μl goat anti-rabbit IgG Alexa 488 conjugate (Molecular Probes, Eugene, OR) diluted 1:250 in PBS for 30 min in the dark. Finally, filters were washed another three times with PBS and mounted on slides in Vectashield (Vector Laboratories, Burlingame, CA). Horizontal and vertical images were obtained with a Bio-Rad MRC-100 laser scanning confocal imaging system (Bio-Rad, Hercules, CA).

**22Na+ Uptake**

Cells were seeded in 24-well plates at a density of 3 × 105 cells/cm2 and grown for 2 d. On the second day, cells were transferred to serum-free medium, followed by an incubation in Cl−-free Krebs-Henseleit bicarbonate (KHB) medium containing 5 mM Hepes/Tris (pH 7.4), 96 mM sodium gluconate, 2 mM potassium gluconate, 1.8 mM calcium gluconate, 1 mM Mg(NO3)2, 2.5 mM sodium pyruvate, and 5 mg/ml gentamicin for 1 to 3 h. Next, Cl−-depleted cells were transferred to 500 μl uptake medium containing 20 mM Hepes/Tris (pH 7.4), 72 mM N-methyl-d-glucosamine-HCl, 48 mM NaCl, 5 mM KCl, 2 mM Na2HPO4, 1 mM CaCl2, 1 mM MgSO4, 0.5 mM ouabain, 100 μM amiloride, 100 μM bumetanide, and 1 μCi per milliliter of 22Na+ and incubated for 15 min at room temperature with or without 100 μM hydrochlorothiazide (HCT). Ouabain was added to prevent Na+ exit via the Na+/K+−ATPase, bumetanide to inhibit the Na+/K+−2Cl− cotransporter, amiloride to block the Na+/H+ antiporter and epithelial Na+ channels, and HCT to inhibit NCC. The uptake reaction was stopped after 15 min by washing the cells 4 times with ice-cold uptake medium. The cells were lysed in 500 μl of 0.1% (w/v) SDS, and radioactivity was counted in a liquid scintillation counter.

**22Na+ Transport**

Cells were seeded on 0.33 cm2 filters (3 × 105 cells/cm2) and grown for 2 d to form a confluent monolayer. Monolayers were
washed with KHB medium containing 110 mM NaCl, 5 mM KCl, 2 mM NaH2PO4, 1.2 mM MgSO4, 1 mM CaCl2, 10 mM sodium acetate, 1 mM L-lactate, 10 mM D-glucose, 4 mM L-alanine, and 20 mM Heps/Tris (pH 7.4). Next, 100 μl KHB medium containing 100 μM amiloride, 100 μM bumetanide, and 0.5 μCi/ml 22Na+ was added at the apical side of the filter. Where indicated, 100 μM HCT was included. At the basolateral side of the cells, 600 μl KHB medium containing 100 μM amiloride and 100 μM bumetanide was added. Sampling (10 μl) from the basolateral side was measured by adding amiloride, 100 μM bumetanide, and 0.5 μCi/ml 22Na+ to the basolateral side, followed by addition of Na+ transport form the apical to the basolateral side was measured by counting added Na+ in a liquid scintillation counter.

**Statistical Analyses**

Values are expressed as mean ± SEM. Statistical significance was determined by t test. Differences in mean with P < 0.05 were considered statistically significant.

**Results**

**Generation of a MDCK Cell Line Stably Expressing hNCC**

To generate a polarized renal epithelial cell line stably expressing hNCC, MDCK cells were transfected with pCB6-hNCC by means of the calcium phosphate precipitation procedure. Transfection was performed in the absence and presence of the specific inhibitor of NCC, hydrochlorothiazide (1 μM HCT). The inhibitor was added to protect the cells from possible deleterious effects as a result of increased Na+ uptake. Transcription of hNCC is driven by a cytomegalovirus promoter. G418-resistant colonies were selected at 10 to 14 d after transfection and tested for hNCC expression by Western blot analysis of total cell lysate. Figure 1 shows seven G418-resistant colonies were selected at 10 to 14 d after transfection and tested for hNCC expression by Western blot analysis of total cell lysate.

**Glycosylation State of hNCC Stably Expressed in MDCK Cells**

Figure 1 shows a broad band of high molecular weight (approximately 120 to 140 kD) and a single band (2 and 7) of lower molecular weight (approximately 110 kD). To assess the glycosylation state of the NCC comprising these bands, total cell lysate of clone 2 was incubated with either endoglycosidase H (EndoH) or N-glycosidase F (PGNaseF). The broad band of approximately 120 to 140 kD completely disappeared after treatment with PGNaseF, leaving a nonglycosylated protein of approximately 100 kD (Figure 2). On the other hand, treatment with EndoH did not affect this broad band. This indicates that hNCC stably expressed in MDCK cells is predominantly complex glycosylated. Lane 4 shows that EndoH treatment resulted in the disappearance of the approximately 110 kD band and the appearance of an approximately 100-kD band. This suggests that the small band of approximately 110 kD reflects the high-mannose glycosylated form of the transporter. The apparent molecular sizes of the NCC expressed in MDCK cells are in good agreement with those reported in oocytes and rat kidney (28,29). Because with increasing passage number the ratio between high mannose and complex glycosylated hNCC changed in favor of the high mannose form, we only used low passage numbers in this study.

**Plasma Membrane Localization of hNCC Stably Expressed in MDCK Cells**

For immunolocalization studies, cells were grown on permeable supports until they formed a confluent monolayer. After mild permeabilization and fixation, cells were stained with anti-hNCC antibody A857 (26). Figure 3 depicts mono-
played a relatively high Na+
expressed at the cell surface. Untransfected MDCK cells dis-
4B). These data demonstrate that the hNCC is functionally
lateral Na+
served in untransfected cells. Similarly, hNCC-MDCK cells
was determined by measuring thiazide-sensitive22Na
uptake in hNCC-MDCK cells to the level ob-
M, 15 min) increased in
P<0.05) increased in
HCT as a function of time (mean of two filters).

Figure 3. Localization of human Na\(^{+}\)-Cl\(^{-}\) cotransporter (hNCC) stably expressed in MDCK cells. Nontransfected MDCK cells (A) and hNCC-MDCK cells (B; Figure 1, clone 2) were grown to confluence on permeable supports. Immunocytochemistry was performed with rabbit anti-hNCC antibody A857 as a primary antibody and goat anti-rabbit IgG Alexa 488 conjugate as the secondary antibody. XY (A and B, top) and XZ (B, bottom) images were obtained by confocal laser scanning microscopy. Nontransfected monolayers show background staining only (A), whereas hNCC-MDCK monolayers show a heterogeneous expression pattern (B, top). The cross section reveals that hNCC is largely expressed in the apical part of the cell (B, bottom).

layers of untransfected MDCK cells (A) and cells of clone 2 expressing hNCC (B). The figure shows that expression of hNCC is clearly heterogeneous. The Z-scan shows a predom-
ant expression to the apical domain of the cell.

Thiazide-Sensitive Na\(^{+}\) Uptake in hNCC-MDCK Cells

The functionality of NCC stably expressed in MDCK cells was determined by measuring thiazide-sensitive \(^{22}\text{Na}\) uptake. Figure 4A shows that \(^{22}\text{Na}\) uptake was significantly increased in cells expressing hNCC as compared with the corresponding untransfected cells (P<0.05). Importantly, HCT (1 \(\mu\)M) reduced Na\(^{+}\) uptake in hNCC-MDCK cells to the level ob-
served in untransfected cells. Similarly, hNCC-MDCK cells cultured on permeable supports exhibited net apical to baso-
lateral Na\(^{+}\) transport that was inhibited by thiazides (Figure 4B). These data demonstrate that the hNCC is functionally expressed at the cell surface. Untransfected MDCK cells displayed a relatively high Na\(^{+}\) uptake (Figure 4A). However, the presence of bumetanide and amiloride in the medium excluded the involvement of the Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter, the Na\(^{+}\)-H\(^{+}\) antiporter, and epithelial Na\(^{+}\) channels. At present, we have no explanation for the relatively high Na\(^{+}\) uptake value in untransfected MDCK cells.

Thiazide-Sensitive Na\(^{+}\) Uptake in hNCC-MDCK Cells is Increased with Forskolin

To determine possible effects of aldosterone, phospho-in-
ositide 3-kinases (PI3Ks) or protein kinases A (PKA), G
(PKG), and C (PKC) on thiazide-sensitive Na\(^{+}\) uptake, hNCC-

MDCK cells were incubated in Cl\(^{-}\)-free medium containing aldosterone (1 \(\mu\)M, 15 min), wortmannin (100 nM, 1 h), forskolin (30 \(\mu\)M, 30 min), cGMP (100 \(\mu\)M, 45 min), and either PMA (1 \(\mu\)M, 15 min) or staurosporine (1 \(\mu\)M, 15 min), respectively. Figure 5 demonstrates that only forskolin evoked a significant change in Na\(^{+}\) uptake. At the concentration used, forskolin readily increases cAMP, leading to rapid activation of PKA (30). NCC activity was not altered by short-term stimulation with aldosterone or short-term activation of PKG or PKC (PMA). Neither was it altered by short-term inhibition of PI3K (wortmannin) or PKC (staurosporine).

Expression of hNCC Is Increased on Long-Term Treatment with PMA and Aldosterone

Long-term (16 h) treatment with 1 \(\mu\)M aldosterone markedly

increased hNCC expression (Figure 6A, upper panel, lane 3). The figure shows that aldosterone preferentially upregulated the complex glycosylated species of hNCC (approximately 120 to 140 kD). In contrast, long-term PMA treatment dramatically increased the expression of both complex and high mannose glycosylated NCC (data not shown). To visualize the effects of aldosterone and PMA on the same blot, significantly less protein was loaded in lane 4 of the gel. Remarkably, this lane shows that PMA predominantly increased expression of the high mannose glycosylated form of hNCC (approximately 110 kD). MDCK cells stably expressing aquaporin 2 (AQP2) were used to investigate the specificity of the effects of aldosterone and PMA. Figure 6A (bottom panel, lanes 3 and 4) shows that both stimuli increased AQP2 expression to the same extent. Note that in this case, equal amounts of protein were loaded onto the gel.
Uptake in hNCC-MDCK Cells Is Increased on Long-Term Incubation with Aldosterone but Not PMA

To determine possible effects of aldosterone on thiazide-sensitive Na$^+$ uptake, MDCK cells stably expressing hNCC were incubated in serum-free medium containing aldosterone for 16 h. Thiazide-sensitive Na$^+$ uptake was measured in the absence and presence of 100 nM aldosterone (HCT), and uptake values were corrected for the number of cells present in the well. During Cl$^-$ depletion, cells were pretreated with aldosterone (1 μM, 15 min), phorbol 12-myristate 13-acetate (PMA) (1 μM, 15 min), cyclic guanosine monophosphate (cGMP) (100 μM, 45 min), staurosporine (1 μM, 15 min), wortmannin (100 nM, 1 h), and forskolin (30 μM, 30 min). For each condition, the uptake value obtained in the presence of aldosterone (basal uptake) was set at 100%, to which the corresponding value obtained in the absence of inhibitor was related. Depicted is the percentage increase. Thiazide-sensitive $^{22}$Na$^+$ uptake was significantly ($P < 0.05$) higher in cells pretreated with forskolin. Except for forskolin (six filters), the values presented are the mean ± SEM of three filters. *Significantly different from hNCC-MDCK (control).

Discussion

Here, we describe for the first time the generation of a high-resistance MDCK cell line stably expressing the human thiazide-sensitive Na$^+$-Cl$^-$ cotransporter. NCC was largely present in the complex glycosylated form and localized to the apical domain of the MDCK cells. The cells exhibited a significant thiazide-sensitive Na$^+$ uptake, which was increased on short-term treatment with forskolin. Moreover, hNCC-MDCK cells cultured on permeable supports displayed a thiazide-sensitive transepithelial Na$^+$ transport from the apical to basolateral compartment. Together, these data show that hNCC-MDCK cells are a good model to study the structural requirements and regulatory aspects of localized trafficking and ion-transporting activity of this cotransporter.
Thus far, studies on the molecular mechanisms underlying the apical sorting process and the regulation of the ion-transport activity of hNCC have been hampered by the lack of an appropriate mammalian expression system. In the past years, many studies have used polarized MDCK cells to functionally express a wide variety of transporter proteins (25,31–33). Here, we report the successful expression of hNCC in this polarized mammalian cell of renal origin. Both low- and high-expressing hNCC-MDCK cell lines exhibited normal growth rates, showing that overexpression of the cotransporter did not affect cell viability. In agreement with this, hNCC-MDCK cells were found to grow equally well in the absence or presence of hydrochlorothiazide. Immunocytochemical analysis revealed that hNCC was present in the apical domain of the cell, with virtually no staining at the basolateral side. This is in agreement with the observation that in rat, NCC is localized predominantly on apical microvilli of cells lining the DCT (12). The observed increase in thiazide-inhibitable Na\(^+\) uptake and transepithelial transport indicates that at least part of the cotransporter is functional and therefore present in the apical membrane. The same observation was reached with a number of other transporters with apical expression in their native cells, including the excitatory amino acid transporter 3 (32), AQP2 (25), and glycine transporter 2 (33).

Western blot analysis revealed that hNCC was present in both the high mannose (approximately 110 kD) and complex glycosylated (approximately 120 to 140 kD) form, whereas the unglycosylated protein, obtained after PG\(\text{NaseF}\) or EndoH treatment, had an apparent molecular size of approximately 100 kD. By use of the *Xenopus laevis* expression system, we (24) and others (29) have previously shown that complex glycosylation is a prerequisite for functional targeting to the plasma membrane. Here, we show that MDCK cells predominantly express the approximately 120- to 140-kD form of the hNCC, with only very little expression of the 110-kD protein. Importantly, the same profile has been reported for mouse kidney cortical membranes (28). The glycosylation pattern was not altered in cells in which the expression of hNCC was moderately increased after long-term stimulation with aldosterone. On the other hand, it was completely reversed when hNCC expression was dramatically increased after chronic treatment with PMA. This suggests that the cellular sorting machinery becomes obstructed at high protein production rates. Alternatively, long-term PMA treatment may lead to inhibition at some step of the sorting process. In contrast to hNCC-MDCK cells, *Xenopus laevis* oocytes predominantly express the approximately 110-kD protein (24,28). This glycosylation pattern is not likely to be due to excessive hNCC production because AQP2, when expressed at similar levels, was hardly detected in the high mannose glycosylated form (34). Therefore, this finding rather suggests that *Xenopus laevis* oocytes lack a chaperone type of protein necessary for proper processing of hNCC. In agreement with this, recent coexpression studies that used *Xenopus laevis* oocytes showed that the glucose-regulated protein 58 (grp58), a chaperone with thiol-dependent reductase activity, associates with NCC and increases NCC-mediated Na\(^+\) uptake (35). The present demonstration of a “native” glycosylation pattern in hNCC-MDCK cells suggests that these cells of distal/collecting tubule origin do possess such a chaperone. Therefore, this model may be of use in developing strategies for improvement of apical sorting of (partially) retained Gitelman syndrome mutants.

To date, little has been learned about the regulation of hNCC. Here, we report that hNCC-mediated Na\(^+\) uptake is stimulated by forskolin, a drug that promotes the adenyl cyclase–catalyzed production of cAMP. In view of the rapidity of the response, the effect of cAMP, acting through PKA, is not likely to be due to increased hNCC expression. Rather, it reflects activation of cotransporters already present on the cell surface and/or their increased shuttling between intracellular vesicles and the apical plasma membrane. In this context, forskolin-induced redistribution from intracellular vesicles to the apical membrane has been demonstrated in MDCK-HRS cells stably expressing AQP2 water channels (25). Of all species studied thus far, only hNCC contains a potential phosphorylation site for PKA (36). This explains the lack of effect of cAMP on thiazide-sensitive Na\(^+\) uptake in *Xenopus laevis* oocytes expressing flounder NCC (20) and suggests that this site is important in cAMP-dependent activation of hNCC-mediated Na\(^+\) transport. At present, it is unclear whether and which hormones or paracrine factors act through cAMP to regulate hNCC in DCT. In rat, DCT expresses prostaglandin EP4 (37) and vasopressin V2 (38) receptors. Moreover, immortalized mouse DCT cells accumulate Na\(^+\) in response to β-adrenergic receptor agonists (39). Finally, microdissected distal tubules from rabbit display cAMP rises in response to calcitonin and vasoactive intestinal peptide in DCT and to parathyroid hormone and isoproterenol in connecting tubule (CNT) (40).

In the long term, aldosterone caused a significant increase in thiazide-sensitive Na\(^+\) uptake. This was accompanied by increased expression of both the high mannose and complex glycosylated form of the transporter, but without changing their relative abundances. Because expression of hNCC is driven by a cytomegalovirus promoter/enhancer, this effect of aldosterone is not likely to be specific. This was confirmed by the observation that aldosterone significantly increased AQP2 expression driven by the same cytomegalovirus promoter/enhancer in MDCK-HRS cells stably expressing this water channel.

Short-term treatment with either PMA or staurosporine did not alter thiazide-sensitive Na\(^+\) uptake in hNCC-MDCK cells. This shows that PKC does not act directly or indirectly on hNCC activity and/or cell surface expression in these cells. In contrast, PMA was found to markedly decrease Na\(^+\) uptake in *Xenopus laevis* oocytes expressing either flounder (20) or rat (21) NCC. This suggests that PKC affects NCC activity and/or trafficking differentially in polarized MDCK cells and oocytes. It should be noted that all NCC sequenced thus far contain putative PKC consensus sites (36). The lack of effect of cGMP and wortmannin indicates that neither PKG nor PI3K play a role in hNCC-mediated Na\(^+\) uptake (35). The present demonstration of a “native” glycosylation pattern in hNCC-MDCK cells suggests that these cells of distal/collecting tubule origin do possess such a chaperone. Therefore, this model may be of use in developing strategies for improvement of apical sorting of (partially) retained Gitelman syndrome mutants.
Taken together, these findings are compatible with a model in which PKA directly or indirectly activates hNCC and/or increases its membrane expression, whereas WNK4 works in the opposite direction.

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